Electrophoretic Separation and Determination of Flavins

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The available chemical, biological and microbiological methods for the determination of individual flavins of biological interest [flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN, riboflavin 5′-phosphate), riboflavin] and of their commonest breakdown products (lumichrome, lumiflavin) have proved unsatisfactory when applied to a mixture of these compounds.

Attempts to separate different flavins in a mixture have been made by previous authors (Crammer, 1948; Hais & Pécakova, 1949; Carter, 1950; Yagi, 1951; Dimant, Sanadi & Huennekens, 1952; Huennekens, Sanadi, Dimant & Schepartz, 1953), using paper chromatography. They did not obtain, however, a complete resolution of all flavins, nor could they determine quantitatively the separated compounds. Siliprandi, Siliprandi & Lis (1954) have recently separated riboflavin, FMN and FAD by paper electrophoresis. To our knowledge no convenient procedure has, however, been reported for separating all the flavins of a mixture.

A method for the separation and estimation of all the flavins normally encountered is described in this communication.

EXPERIMENTAL

Materials. Riboflavin was a commercial preparation obtained from H. La Roche. FMN was prepared according to Viscontini, Ebnoether & Karrer (1952), FAD according to Siliprandi & Bianchi (1955), lumiflavin according to Warburg & Christian (1932), and lumichrome according to Karrer, Salomon, Schopp & Schlittler (1934).

Electrophoresis of flavins

Paper electrophoresis. A mixture containing not more than 7 μg. each flavin was applied in one spot to a strip (6 cm. × 25 cm.) of Munktell 20 paper, at a distance of 10 cm. from the cathode. The paper was then moistened with a buffer solution, except where the mixture had been applied, and lightly pressed between two sheets of filter paper to remove excess of liquid. The electrophoresis was run in sodium acetate buffer, pH 5·1, $I = 0{\cdot}05$. A potential of 380 v was applied for 6 hr., giving a current of 1·2 mA/cm. width.

For qualitative electrophoresis the paper was placed between two glass plates firmly clamped together. The ends of the strips were cut off, at the end of the run, in order to prevent the spread of the liquid from the buffer solution into the paper, and the paper was dried in a stream of hot air. The spots were located with a Mineralight short-wave ultra-

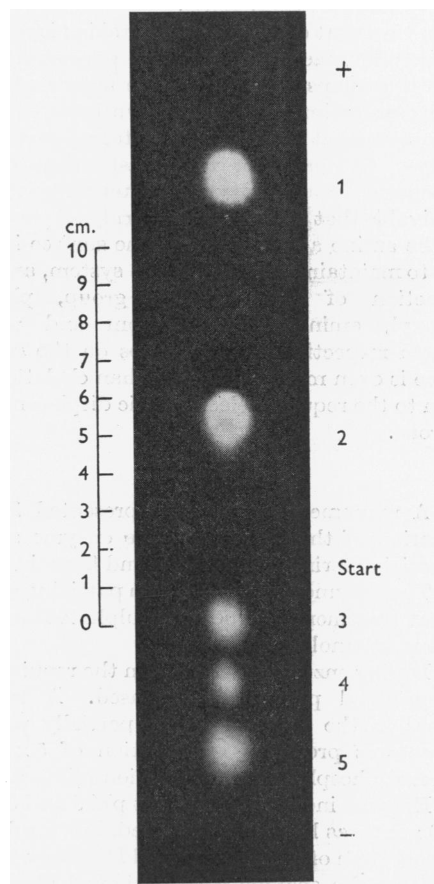

Fig. 1. Separation of a mixture of flavins by paper electrophoresis: sodium acetate buffer, pH 5·1, I 0·05; 1·2 mA/cm. during 6 hr. 1, FAD; 2, FMN; 3, lumiflavin; 4, lumichrome; 5, riboflavin. The photograph was taken in ultraviolet light, with panchromatic plates and a green filter on the objective; exposure time, 45 sec.

violet lamp, a filter SL 2537 (Ultraviolet Products Inc., South Pasadena, California) being used, and their identification was carried out by reference to the spots obtained from a known mixture in a parallel run.

Spots due to riboflavin and FMN show a bright-yellow, and the spot due to FAD shows a pale-yellow, fluorescence. The spot due to lumiflavin shows a greenish yellow, and that due to lumichrome a bright sky-blue, fluorescence.

The separation of the most important flavins is shown in Fig. 1. FAD and FMN migrate towards the anode, FAD being twice as fast as FMN; riboflavin, lumiflavin and lumichrome migrate in decreasing order of mobility towards the cathode.

No breakdown of the pure compounds was observed when these were run separately. For this reason, and because the spots are sharply defined without tailing, the electrophoretic separation can be usefully applied to the quantitative estimation of the different flavins in a mixture.

For this purpose an accurately measured volume of the solution to be analysed was applied on the paper from an Agla micrometer syringe (Burroughs Wellcome Ltd.). The electrophoresis was run with the paper hanging free in a closed chamber; all the other described conditions were maintained with the exception of drying the paper, which was done in a fume cupboard at room temperature. The spots were detected and identified as described above.

Strips of equal size and shape, each containing one spot, were cut out and eluted with water as described by Brimley & Barret (1953), at a flow rate of 1 ml./hr.; in each case 8 ml. eluate were collected in a graduated tube. This volume was usually sufficient to elute the substances completely, but occasionally a larger volume was collected as a check on recovery.

The fluorescence of the eluate was determined in a Coleman 12 B photofluorimeter (Coleman Instruments Inc., Maywood, Illinois), the fluid collected under the same conditions from a similar piece of filter paper from the same strip being used as a blank. The amount of flavin was estimated by comparing the fluorescence with that of a known solution of the pure compound.

However, from the measurement of fluorescence the recovery of riboflavin, FMN and FAD appeared to be incomplete. For this reason the recovery for these flavins was also estimated by measuring the extinction coefficients of the eluates at 260 and 450 mμ. A concentration of flavins suitable for spectrophotometric measurements was obtained by depositing mixtures containing up to 50 μg. of each of these compounds in a line on the filter paper. Results reported in Table 1 show that when determined spectrophotometrically the recovery of riboflavin FMN and FAD, as well as that of the other flavins, is complete.

Cellulose-column electrophoresis. By use of cellulose-column electrophoresis a resolution of larger amounts of flavins was obtained. The apparatus and the technical details were according to Flodin & Porath (1954).

Table 1. *Elution of flavin mixtures separated by paper electrophoresis*

Electrophoresis on Munktell 20 paper, using sodium acetate buffer, pH 5·1, $I = 0·05$; potential applied 380 v, giving a current of 1·2 mA/cm., for 6 hr. Eluent, water. Data reported under *A* are calculated from measurements of the fluorescence of the eluates; data under *B* are calculated from measurements of the extinction coefficients at 260 and 450 mμ.

Substance	Amount applied (μg.)	Amount recovered (μg.)		Recovery (%)	
		A	*B*	*A*	*B*
Riboflavin	0·5	0·24	—	48	—
	2·5	1·23	—	49·6	—
	5	2·45	—	49	—
	50	24·7	53·2	49·4	106·4
FMN	0·5	0·29	—	58	—
	2·5	1·44	—	57·7	—
	5	2·93	—	58·4	—
	50	29·1	56·7	58·2	113·4
FAD	0·9	0·64	—	71·1	—
	1·8	1·28	—	71·2	—
	4·5	3·17	—	70·7	—
	7·2	5·14	—	71·4	—
	50	35·3	51·4	70·6	102·8
Lumiflavin	0·5	0·48	—	96	—
	2·5	2·44	—	97·6	—
	5	48·6	—	97·2	—
Lumichrome	0·5	0·47	—	94	—
	2·5	2·31	—	92·4	—
	5	46·6	—	93·2	—

Sodium acetate buffer, pH 5·1, *I* 0·05, or 0·05 M ammonium formate, pH 4·8, was used. A mixture of flavins containing not more than 1·5 mg. each compound in 4 ml. buffer was put on the top of the column (50 cm. × 3 cm.) of cellulose powder (Whatman B quality, standard grade).

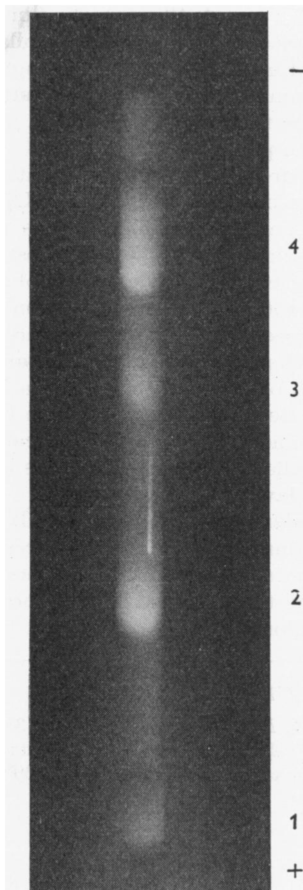

Fig. 2. Electrophoretic separation of a mixture of flavins on a cellulose column (ammonium formate buffer, pH 4·8, 0·05 M; 50 mA for 14 hr.). 1, FMN; 2, riboflavin; 3, lumiflavin, 4, lumichrome. The photograph was taken in ultraviolet light, with panchromatic plates and a green filter on the objective; exposure time: 40 sec.

Before the current was applied the mixture was displaced 15–20 cm. down the column, by allowing a sufficient amount of buffer to flow through the column. The top of the column was then connected to the cathode, the bottom to the anode and a potential of 520 v applied for 14 hr.

The separation of mixtures of flavins by column electrophoresis is shown in Figs. 2 and 3.

On completion of the run the column was disconnected from the electrophoresis apparatus and placed on a fraction collector. The elution was performed with the same buffer as that used for the electrophoresis and the flow rate was adjusted to 25 ml./hr. The effluent was collected in 8 ml. fractions and the extinction coefficients of these solutions were measured in a Beckman model D.U. spectrophotometer at 250 and 450 mμ. The identification of the compounds was carried out with the help of paper electrophoresis as previously described. When necessary, the fractions to be analysed were evaporated under reduced pressure, at 35°, until a suitable concentration for paper electrophoresis was reached. When it was necessary to concentrate the eluate ammonium formate buffer was used, since the constituents are volatile and a smaller amount of buffer ions was left in the concentrate.

Quantitative recoveries have been obtained in these experiments.

Differential adsorption of flavins on cellulose

During the preliminary experiments a differential adsorption of some of the flavins on cellulose was noticed. Fig. 4 shows the chromatographic separation of a mixture containing 0·5 mg. FMN, 0·25 mg. riboflavin, 0·1 mg. lumichrome and 0·2 mg. lumiflavin on a column (50 cm. × 3 cm.) packed with cellulose powder (Whatman B quality, standard grade). The same acetate buffer as in the electrophoresis experiments was used as solvent. This procedure, however, does not permit the separation of FAD from FMN.

All the experiments were carried out as far as possible in the absence of light, so as to prevent the breakdown of flavins which are highly photolabile compounds.

DISCUSSION

The chromatographic procedures reported by previous authors (Crammer, 1948; Hais & Pécakova, 1949; Carter, 1950; Yagi, 1951; Dimant *et al.* 1952; Huennekens *et al.* 1953) for the separation of flavins are not altogether satisfactory. They do not allow a complete resolution of mixtures containing several riboflavin derivatives, the separation of the individual compounds is not sharp, and the R_F values of some of the compounds are rather variable.

The paper-electrophoresis procedure described here permits a sharp and readily reproducible separation of riboflavin and its derivatives, and can therefore be considered as the most suitable technique for the analysis of flavin mixtures in microgram quantities so far proposed. Paper electrophoresis has, moreover, a much higher resolving power than paper chromatography.

Indeed traces of FMN contaminating FAD solutions, and not revealed by paper chromatography, are readily resolved as a distinct spot by paper electrophoresis. Hydrolysis of FAD during the electrophoretic run can be excluded as a reason for this second spot, since no breakdown of flavins has been observed when pure compounds were run separately.

Paper electrophoresis has been successfully applied to the quantitative determination of each component of a flavin mixture. A total recovery has been obtained, although the fluorescence readings on the eluted samples are lower than expected. This may be related to a quenching of the fluorescence during the electrophoresis. A quenching of fluorescence of flavins in saline solu-

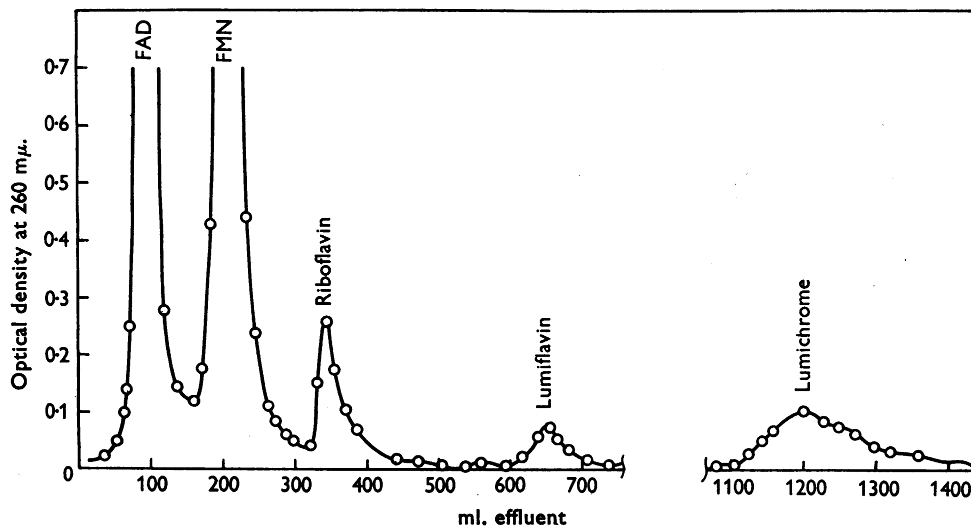

Fig. 3. Elution curves of a mixture of flavins separated by cellulose-column electrophoresis (sodium acetate buffer, pH 5·1, *I* 0·05; 520v for 14 hr.). FAD, 0·3 mg.; FMN, 0·55 mg.; riboflavin, 0·08 mg.; lumiflavin, 0·1 mg.; lumichrome, 0·15 mg.

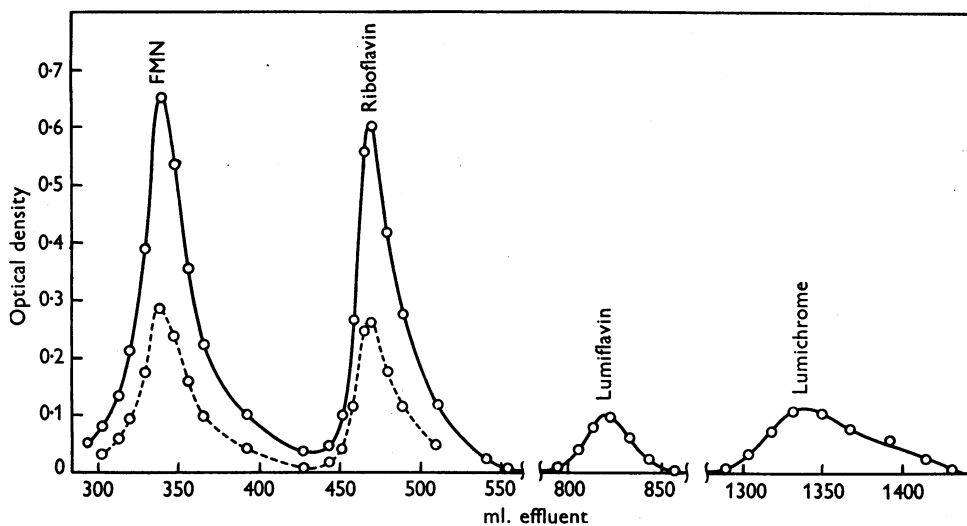

Fig. 4. Elution curves of a mixture of flavins separated by chromatography on cellulose column. Solvent: sodium acetate buffer, pH 5·1, *I* 0·05. Flow rate 30 ml./hr. FMN, 0·5 mg.; riboflavin, 0·25 mg.; lumichrome, 0·12 mg.; lumiflavin, 0·08 mg. —, 260 mμ.; - - - -, 450 mμ.

tions has already been pointed out by Bessey, Lowry & Love (1949) and Weber (1950). However, since the quenching of fluorescence is constant, fluorescence measurements can be used, with an error of ± 2 %, for the determination of the eluted flavins. Fluorimetry has been preferred, as it is 30 times more sensitive than spectrophotometry at 260 mμ. and 100 times more sensitive than colorimetry (Hegsted, 1954).

The separation of larger amounts of each compound has been obtained by using cellulose-column electrophoresis; this method can therefore be employed for micropreparative purposes.

A differential adsorption on cellulose of some flavins has been observed. As will be seen from the elution curves in Fig. 4, the degree of adsorption increases in the following order: FAD (= FMN), riboflavin, lumiflavin, lumichrome. Consequently, when displaced 15 cm. down the column before starting the electrophoresis, the flavin mixture undergoes a partial resolution, its components being distributed in distinct zones. When the current is applied the flavins are at different levels in the column and so start their electrophoretic run from different positions. This fact does not hinder the electrophoretic separation on a cellulose column; however, the distribution pattern of the separated compounds is different from that obtained on paper, where all the substances start from the same place.

The methods described here have been successfully applied to the determination of flavins in biological materials.

SUMMARY

A method for the separation and quantitative determination of riboflavin and its derivatives (flavin mononucleotide, flavin-adenine dinucleotide, lumichrome and lumiflavin) by paper electrophoresis is described. A micropreparative separation of the same compounds has been achieved by means of cellulose-column electrophoresis.

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The Relation between Thiamine, Biotin and Tryptophan Metabolism, Studied in the Rat

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Previous papers (Dalgliesh, 1952; Charconnet-Harding, Dalgliesh & Neuberger, 1953) have shown that useful information on the relationship of B-vitamins to tryptophan metabolism may be obtained by examining urinary metabolites excreted after ingestion of tryptophan by vitamin-deficient animals. The present paper describes a continuation of this work with an elaboration of the technique. The effects of deficiencies of thiamine and biotin have been investigated. Thiamine deficiency has been known for some time to decrease the conversion of tryptophan into nicotinic acid derivatives by the rat (Junqueira & Schweigert, 1948), but there has been no evidence to show at what stage in the conversion thiamine functions. A preliminary survey of other B-vitamins suggested that pantothenic acid, nicotinic acid and biotin had no effect on tryptophan metabolism